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THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 57, No. 5
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THE INCORPORATION OF ^{14}C-LEUCINE INTO THE PROTEINS OF PLUCKED WOOL FOLLICLES*

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ABSTRACT

Protein synthesis in wool follicles was studied by incubating plucked wool fibers at 37° C in Krebs-Ringer-phosphate buffer containing ^{14}C-leucine and glucose. The incorporation of ^{14}C-leucine by the plucked fibers was maintained for 12–24 hours in the presence of glucose. Autoradiographs showed that the incorporated radioactivity was mainly confined to the bulb region of the fiber. Initially most of the ^{14}C was present in wool root proteins which were dissolved or dispersed by 8M urea. During incubation these proteins became increasingly insoluble in 8M urea. After 24 hours' incubation the wool roots appeared completely urea-resistant and almost all of the incorporated ^{14}C was present in urea-insoluble material. This change in solubility was probably due to non-enzymatic oxidation of thiol groups and was catalyzed by cupric ions in the presence of atmospheric oxygen.

Electrophoresis of the wool root proteins synthesized *in vitro* showed that ^{14}C-leucine was mainly incorporated into low-sulfur keratin proteins. Despite the apparent difficulties in maintaining the urea-solubility of the wool roots *in vitro*, this synthesis of low-sulfur protein in the bulb region of the plucked fibers indicates that keratin biosynthesis can be satisfactorily studied *in vitro* by this method.

Biochemical and electron microscopic investigations of wool keratin synthesis have shown that the microfibrillar component, consisting of proteins poor in sulfur, is mainly synthesized in the follicle bulb region, while the matrix component, composed of sulfur-rich proteins, is formed in the keratogenous zone of the fiber (1, 2, 3). The development of cell-free protein synthesizing systems, prepared from homogenates of wool or hair roots, has enabled investigation of the protein synthetic pathways of the follicle tissue to be made (4, 5, 6, 7). However, the relationship of such protein synthesis to the formation of keratin has not yet been determined.

Recent investigations have shown that the oxidative metabolism of follicles may be easily studied by incubating plucked fibers *in vitro* in an appropriate medium (8). Other aspects of follicle metabolism and keratin synthesis could also be conveniently investigated using such a simple *in vitro* system. We have examined the application of a similar procedure for studying wool follicle protein synthesis *in vitro*. Adult sheep have the advantage for such studies in that most of their wool follicles are in the anagen VI stage, with a continuous fiber growth phase lasting many years. The harvesting of follicle material by plucking was used in preference to other methods such as wax-sheet removal (9), follicle dissection (10), stripping or latex epilation (6, 7). With these latter methods it was difficult or impossible to remove follicle material rapidly from live sheep. For wax-sheet harvesting it is necessary to kill the animal prior to pouring molten wax onto the skin, and time is required for the wax to set before the follicle tissue can be removed. Follicle dissection is too slow and tedious to be used extensively, and although material can be obtained from live animals by stripping or latex epilation, at least several hours are required for the adhesives to set.

As well as facilitating the transfer of wool roots to an incubation medium with the minimum possible delay, the plucking technique has other advantages. Because of the ease of detecting ^{14}C in plucked wool immersed directly in a scintillation solution (11), the extent of incorporation of a ^{14}C-amino acid by incubated fibers may be readily determined. By arranging the experiment so that each wool root has a comparatively long, fully keratinized fiber attached to

Received February 27, 1971; accepted for publication June 8, 1971.
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it, the proportion of *material* dispersed by a reagent such as 8M urea (1) is negligible. Thus, the proportion of incorporated *radioactivity* removed by 8M urea can be easily calculated from the reduction in counting rate per unit mass of fiber. In the studies reported here, we have examined the conditions required for the *in vitro* incorporation of ^{14}C-leucine into proteins of plucked wool fibers. The site of ^{14}C-leucine incorporation and the nature of the labeled proteins synthesized *in vitro* have also been investigated.

MATERIALS AND METHODS

Experimental animals. Two adult English Leicester wethers were used. Each sheep received a daily ration (10 g/kg body weight) consisting of equal parts of lucerne chaff and oat grain.

Reagents. All reagents used were analytical grade. The L-^{14}C-leucine (U) (170 mCi/m mol) and n-(1-^{14}C)-hexadecane (0.97 μCi/g) were obtained from the Radiochemical Centre, Amersham, England.

Preparation of tissue. Seven days prior to each experiment the wool was uniformly clipped from a 20 x 20 cm area on the side of the sheep. On the day preceding each experiment the area was washed with light petroleum and with 70% ethanol to remove the surface dirt and wax, and then covered with a cloth until required. Using surgical forceps, the samples were plucked at random from the prepared area as rapidly as possible and immediately placed in the incubation medium. Each sample weighed 30–60 mg and comprised about 12 separate bundles of wool.

Incubation procedure. The samples were incubated in weighed glass vials, of 20 ml capacity, containing 5 ml Krebs-Ringer-phosphate buffer (pH 7.3), uniformly labeled ^{14}C-leucine (0.08 or 0.32 μCi) and glucose (2 mg/ml). The medium was saturated with oxygen/carbon dioxide (95:5 v/v) just before use and the samples were then incubated under air. For incubations longer than 6 hours' duration, the medium was sterilized using a Seitz filter and both streptomycin (50 μg/ml) and penicillin (100 μg/ml) were added to the medium. After incubating the samples at 37° C for various times, the medium was removed and the sample was then given four 15-minute washes, at room temperature (20° C), with 10 ml of a solution of unlabeled leucine (7.6 mM) and NaF (4.8 mM). Preliminary experiments showed that less than 8% of the ^{14}C incorporated was removed by further washes with this solution.

The samples were then washed successively with water, ethanol and light petroleum (b.p. 60–80° C), dried, weighed and the ^{14}C content determined by liquid scintillation counting.

Extraction of incubated wool roots. Part of each incubated and washed wool sample was extracted with 8 M urea containing iodoacetate (0.073 M) and EDTA (0.01 M) buffered at pH 8.6 with Tris (0.25 M) until the solution was —SH negative (12). The samples were then washed with water, ethanol and light petroleum and their ^{14}C content determined as above.

Autoradiography. Samples of incubated plucked fibers, both before and after extraction with 8 M urea, were stained with hematoxylin, eosin and picric acid, and autoradiographed using Ilford Industrial B X-ray film as described by Downes, Clarke and Dagg (13).

Urea-solubility of wool roots. Plucked samples were first "pulse-labeled" with ^{14}C-leucine by incubating them in labeled medium for 1.5 hours. Preliminary experiments had shown that pre-incubation did not alter the rate of ^{14}C incorporation by samples during subsequent incubations. They were then washed several times with NaCl (0.9% w/v) before being reincubated in unlabeled medium. The conditions of reincubation were altered by increasing the temperature or by the addition of cupric ions (10^{-4} M), disodium EDTA (10^{-2}, 10^{-3} M) or NaF (5 mM) to the medium. The proportion of incorporated ^{14}C which was retained in the fiber after 8 M urea extraction was measured and taken as an index of the resistance of the wool root to urea.

Analysis of wool root extracts. The 8 M urea extracts from incubated wool roots were centrifuged, dialyzed exhaustively against water (18/32 Visking tubing), and then treated with TCA (10% w/v) at 0° C for 30 minutes. In some experiments, the TCA-insoluble material was further extracted with TCA (10% w/v) at 90° C, or with 1N NaOH followed by cold TCA (10% w/v), or dissolved in 0.1N NaOH, 0.2 M thioglycollic acid and then reprecipitated with cold TCA. In each case the remaining insoluble precipitate was washed successively with acetone, ethanol, ethanol-ether (1:1), ether and air dried. Samples of the various soluble fractions were taken for liquid scintillation counting. The final residue was redissolved in 1N NaOH and its ^{14}C content was also determined.

Gel-filtration of wool root extract. Samples of the urea extracts from wool roots which had been incubated for 6 hours were fractionated using Sephadex G25 (medium) which had been equilibrated with 8 M urea. The column (1 x 40 cm) was eluted with 8 M urea. The absorption of the eluate at 254 mμ was recorded continuously with an LKB Uvicord, and the radioactivity was monitored with an anthracene scintillation flow-cell, a ratemeter (Ekco Type N600A) and recorder (Honeywell).

Electrophoresis of wool root proteins. The urea-soluble proteins extracted from samples after a 5-hour incubation in ^{14}C-leucine medium were recovered by dialysis and freeze-drying. Starch-gel electrophoresis of the freeze-dried samples was carried out in 8 M urea solutions at pH 8.6 using the procedures described by Ferguson and Wallace (14) and Ferguson (15). Freeze-dried preparations of carboxymethylated low-sulfur and high-

sulfur proteins previously obtained from wool (2) were simultaneously separated by starch-gel electrophoresis.

The gels were stained with nigrosin, sliced into strips and the optical density of each strip measured with a Joyce-Loebl densitometer. The strips were then cut into segments, dried, combusted and the ^{14}C content determined by liquid scintillation counting (11).

Measurement of radioactivity. Radioactivity in the plucked samples was routinely determined with the samples directly immersed in a liquid scintillation solution (16). The counting efficiency was measured by liquid scintillation counting of representative combusted samples (11) and subsequent calibration of the results with those obtained with known amounts of n-(1-^{14}C)-hexadecane.

The radioactivity in the various aqueous solutions was determined after diluting them to 7.5 ml with H_2O and adding 10 ml of a scintillation solution which contained 7 parts of a toluene solution (toluene 1 liter, PPO 4 g, POPOP 0.1 g) and 6 parts of Triton X-100 by volume. Packard Liquid Scintillation Spectrometers (Models 3324, 3375) were used to measure the radioactivity.

RESULTS

Microscopic examination of the plucked wool fibers showed that about 80% had broken off in the region of the follicle bulb; the rest had been detached at varying levels above this. Inner root sheath material was also removed with many of the fibers. Electron microscopic observations showed that fragments of the outer root sheath were also attached to the plucked fibers.

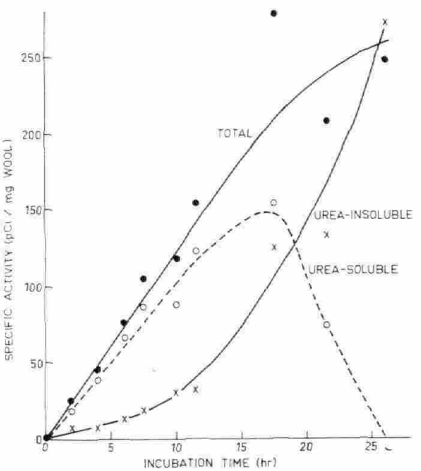


FIG. 1. ^{14}C-leucine incorporation into plucked wool fibers during incubation at 37° C in Krebs-Ringer-phosphate buffer solution containing glucose, pH 7.3. The amount of ^{14}C in the urea-soluble fraction was obtained by subtracting the activity present in the urea-insoluble residue from the total activity of the plucked sample. Each result is the mean for three samples.

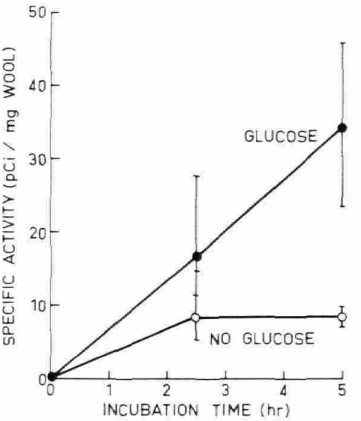


FIG. 2. The effect of glucose (2 mg/ml) on the incorporation of ^{14}C-leucine (0.08 μCi) by plucked wool fibers. The vertical lines indicate the range of results obtained from four samples. The horizontal bars on the lines at 2.5 hours indicate that there was an overlap in the results for these samples.

Incorporation of ^{14}C-leucine. The rate of incorporation of ^{14}C into the plucked wool was approximately constant during the first few hours of incubation (Fig. 1). In this experiment about 0.3% of the ^{14}C-leucine in the medium was incorporated hourly by the plucked wool samples. Repeated experiments using wool plucked from the same sheep at intervals of several weeks showed that the mean rate of incorporation, though linear during the first 12 hours on each occasion, varied widely. In a series of 7 experiments using wool from the same sheep the rate of incorporation ranged from 13 to 38 pCi/mg clean dry wool per hour (Table I). The results for individual samples in each experiment also showed large variations (Fig. 2; note standard errors in Table I). The incorporation of ^{14}C continued during incubations lasting longer than 12 hours, but the results were then more variable. In most experiments, therefore, the incubation times were restricted to less than 12 hours.

TABLE I

Variation in the incorporation of ^{14}C-leucine into wool

Sample	Medium	Sheep	Experiment	Incubation time (hr)	Rate of incorporation (pCi/mg/hr)	Number of samples
Plucked	+ Glucose	1	1	1–5	24 ± 3 (S.E.)	6
			2	4	18 ± 2	10
			3	2–12	23 ± 3	10
			4	5	38 ± 4	4
			5	2–12	13 ± 1	24
			6	1.5	25 ± 1	36
			7	3–5	13 ± 2	6
		2	8	5	27 ± 4	4
			9	3–5	12 ± 2	4
Plucked	− Glucose	1	5	6	4.8, 5.5, 7.7	3
		2	8	5	6.4 ± 0.5	4
Plucked, boiled	+ Glucose	1	3	11	2.3, 1.9	2
Plucked	+ Glucose	1	1	1–5	3.1 ± 0.5	6
	+ NaF (5 mM)	1	5	5	1.7	1
				10	1.4	1
Clipped	+ Glucose	1	1	1–5	1.6 ± 0.2	6
		1	3	11	0.5, 0.6	2

Wool (30–60 mg) was incubated in Krebs-Ringer-phosphate buffer (pH 7.3; 37° C) containing ^{14}C-leucine (0.32 μCi/5 ml) and glucose (2 mg/ml). After incubation the samples were washed and the ^{14}C content determined as described in Materials and Methods.

During the first few hours of incubation most of the ^{14}C was present in the urea-soluble fraction (Fig. 1). However, with longer incubation times, an increasing proportion of the incorporated ^{14}C was found in the urea-insoluble residue; after 24–26 hours incubation, less than 20% of the ^{14}C was extractable with 8 M urea (Fig. 1).

When glucose was omitted from the medium, the incorporation of ^{14}C by the plucked fibers was reduced and ceased after several hours (Fig. 2; Table I). When plucked wool was boiled prior to incubation or when sodium fluoride (5 mM) was present in the medium, some ^{14}C-leucine was incorporated, but the amount was only 10% or less of that observed with plucked wool in the presence of glucose (Table I). Even less ^{14}C-leucine was incorporated into clipped wool during incubation in the labeled medium.

The ability of plucked wool to incorporate ^{14}C-leucine was considerably reduced when a delay occurred between plucking and incubation (Fig. 3). When the plucked samples were left exposed to the air at room temperature (20° C) for 20 minutes or more prior to incubation, the amount of incorporated ^{14}C-leucine was negligible, being similar to that observed with clipped wool. Similar results were obtained in three separate experiments.

Autoradiography. Autoradiographs showed that about 60% of the plucked fibers had incorporated ^{14}C-leucine. The radioactivity was mainly confined to the bulb region, with small amounts in the precortex (Fig. 4). Of the wool roots which failed to incorporate ^{14}C-leucine, about half lacked follicle bulb tissue. The distribution of radioactivity was similar in all samples incubated for varying times up to 24 hours. Occasionally, some ^{14}C was detectable in the inner root sheath material attached to the plucked fiber.

Extraction of freshly plucked samples with 8M urea dispersed most of the unkeratinized portion of the wool root (Fig. 5a). Similar results were obtained when plucked samples were exposed to the atmosphere for up to 17 hours prior to extraction. However, the unkeratinized portion of fibers which had been incubated for several hours was *not* completely dispersed by 8M urea. The insoluble remnants of the bulb and precortex remaining after extraction retained detectable amounts of radioactivity (Fig 5b). After 24 hours' incubation, the wool roots

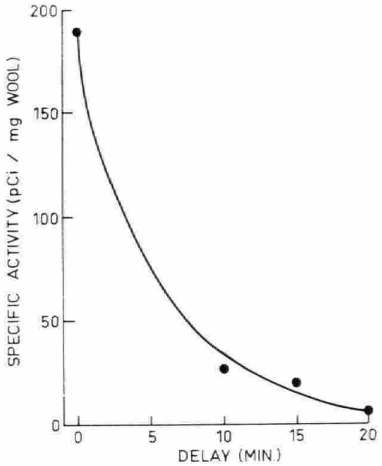


FIG. 3. The effect of delays of various times between plucking and incubation on the incorporation of ^{14}C-leucine by fibers during a 5-hour incubation.

appeared completely urea-resistant (Figs. 1 and 5c).

Factors influencing the urea-solubility of wool roots. Higher incubation temperatures or the addition of cupric ions to the medium resulted in a rapid increase in the stability of the wool roots towards 8M urea (Figs. 6 and 7). In contrast, the presence of EDTA decreased the rate of formation of urea-insoluble material. The addition of NaF to the medium did not affect the subsequent solubility of the wool roots in 8M urea (Fig. 7).

Incubation of plucked fibers in ^{14}C-leucine medium containing cupric ions (10^{-4} M) or disodium EDTA (10^{-2} or 10^{-3} M) showed that although the amount of ^{14}C incorporated was unaltered by the addition of cupric ions, it was considerably reduced in the presence of EDTA (Table II).

Fractionation of 8M urea extract. Dialysis of the urea extracts from the labeled wool roots resulted in a negligible loss of ^{14}C (Fig. 8). Simi-

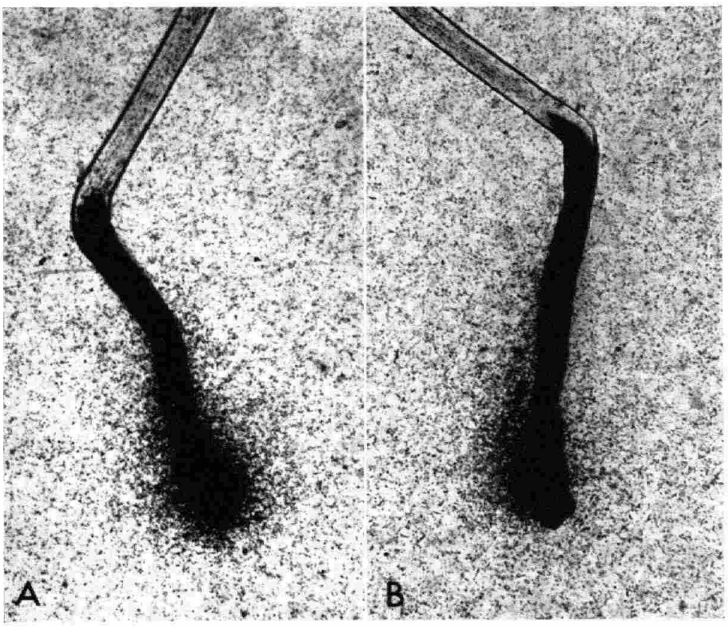


FIG. 4. Autoradiographs of plucked fibers incubated for (a) 4 hours, and (b) 8 hours in ^{14}C-leucine medium. The fiber has been displaced in the 8 hour autoradiograph so that the distribution of radioactivity can be seen clearly. × 90.

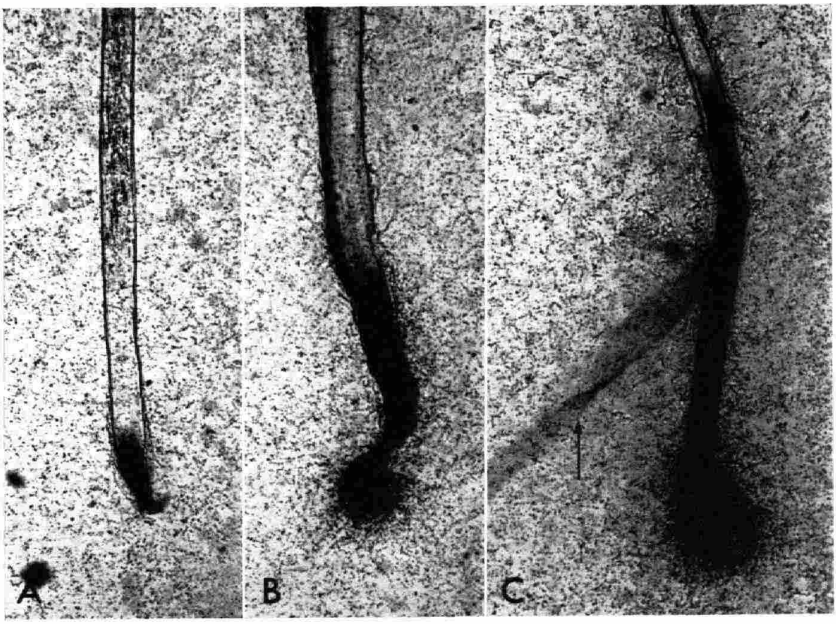


FIG. 5. Autoradiographs of fibers extracted with 8M urea after incubation in ^{14}C-leucine medium for (a) 0 hours, (b) 8 hours and (c) 24 hours. Urea removed most of the unkeratinized portion of the fiber after 0 hours incubation (a). During incubation this portion became increasingly urea-resistant (b, c). Radioactivity was also present in the urea-insoluble residue. The inner root sheath (arrow) shows no apparent incorporation of ^{14}C-leucine. × 90.

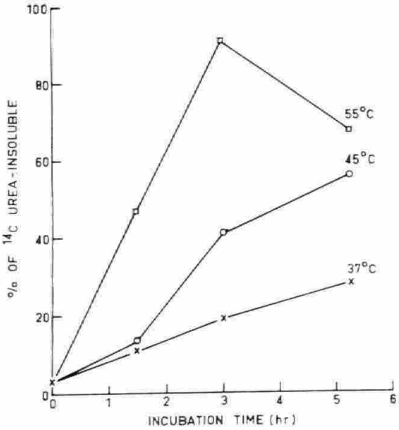


FIG. 6. Effect of incubation temperature on the urea-solubility of wool roots. The proportion of incorporated ^{14}C which was retained by the plucked fibers after 8 M urea extraction was taken as an index of stability.

lar results were obtained from samples which had been incubated for 1, 3 and 5 hours. Only small proportions of the ^{14}C-material were soluble in either cold or hot TCA (10% w/v), or in cold TCA after pre-treatment with either 1N NaOH or 0.1N NaOH/0.2 M thioglycollic acid. The largest proportion of the incorporated ^{14}C (68 to 88%) was found in the insoluble protein remaining after acetone extraction. Fractionation, on Sephadex G25, of the urea extract from samples incubated for 6 hours confirmed that a large proportion (70%) of the radioactivity was confined to material of high molecular weight.

Electrophoresis of wool root proteins. Electrophoretic separation of the 8 M urea-soluble proteins extracted from fibers after a 5-hour incubation showed that both low-sulfur and high-sulfur proteins were present in the extract, although the leading band of the high-sulfur proteins was not very distinct (Fig. 9). Measurement of the radioactivity in the gel after electrophoresis indicated that most of the ^{14}C wa

confined to the low-sulfur protein region of the gel while about 10% of the radioactivity was found in the bands corresponding to the high-sulfur proteins (Fig. 9). About 30% of the ^{14}C remained at the gel origin.

DISCUSSION

The results of these investigations show that plucked fibers incorporated ^{14}C-leucine *in vitro* for periods up to 26 hours using a simple incubation medium. Initially most of the incorporated ^{14}C-leucine was present in the urea-soluble proteins of the wool root. The removal of fibers by plucking proved to be an effective and quick method for obtaining the metabolically active cells of the wool root and these cells continued

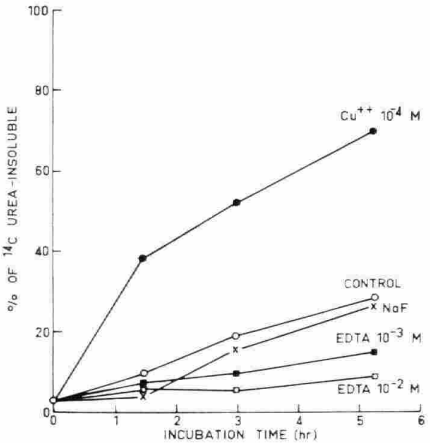
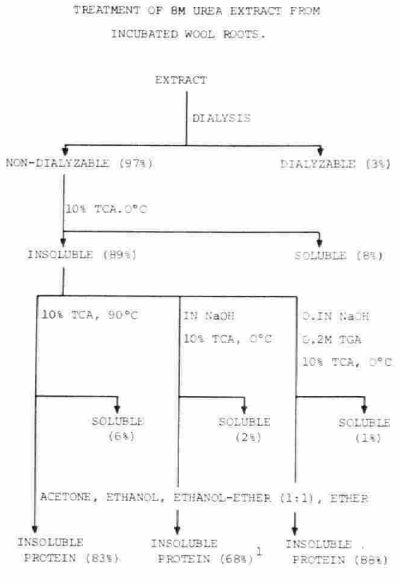


FIG. 7. The influence of various media on the urea-solubility of wool roots. Plucked fibers were "pulse-labeled" with ^{14}C-leucine, then reincubated for various times in unlabeled medium (control), or in unlabeled medium containing NaF (5 mM), $CuSO_4$ (10^{-4} M) or EDTA (10^{-2}, 10^{-3} M) prior to 8 M urea extraction.



1 Portion of the ^{14}C material was also removed during acetone extraction.

FIG. 8. The percentage of ^{14}C in the various fractions obtained during the isolation of protein from the 8 M urea extracts of wool roots. Similar results were obtained with wool which had been incubated for 1, 3 or 5 hours in ^{14}C-leucine medium.

TABLE II

Incorporation of ^{14}C-leucine into plucked wool

Medium	Rate of incorporation (pCi/mg/hr)	
	Experiment 1	Experiment 2
KRP	9.5 ± 0.4 S.E. (27)	25 ± 1 S.E. (36)
KRP + Cu^{++}, 10^{-4} M	—	20, 22
KRP + EDTA, 10^{-2} M	0.8, 1.0, 0.8	1.6, 1.6
KRP + EDTA, 10^{-3} M	3.0, 2.1, 1.9	—

In two separate experiments, wool (60 mg) was incubated at 37° C for 1.5 hours in Krebs-Ringer-phosphate buffer (KRP), pH 7.3, containing glucose (2 mg/ml) and ^{14}C-leucine (0.32 μCi/5 ml). After incubation the samples were washed and the ^{14}C content determined as described in Materials and Methods. The number of samples incubated in KRP alone is shown in brackets. The other results are for individual samples.

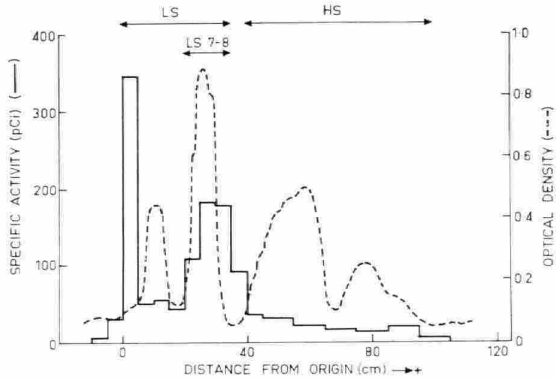


FIG. 9. Densitometer tracing and distribution of radioactivity of a starch-gel electrophoretogram of wool root proteins extracted from plucked fibers after 5 hours' incubation in ^{14}C-leucine medium. The regions marked LS and HS indicate the position of bands characteristic of the low-sulfur and high-sulfur proteins of wool, respectively. The region LS 7–8 corresponds to the major components of the low-sulfur proteins (24).

to synthesize protein *in vitro*. The presence of fibers detached at varying levels above the bulb must, however, contribute to the variation between samples in ^{14}C-leucine incorporation. The inner and outer root sheath material removed with the plucked fibers showed little biosynthetic activity.

Bacterial contamination of the medium during prolonged incubations was minimized by the addition of antibiotics. The small amount of radioactivity incorporated by clipped wool, or by plucked wool after exposure to air for 20 minutes confirmed that the results obtained were not due to bacterial activity.

The presence of glucose was found to be necessary for the incorporation of ^{14}C-leucine to continue for more than several hours, although incorporation was sustained for a brief time in the absence of glucose. Metabolic studies (8, 10) have shown that follicles utilize glucose via the glycolytic and tricarboxylic acid pathways, the pentose cycle also being extremely active. The energy requirements for the prolonged incorporation of labeled amino acid into proteins by follicle cells *in vitro* are presumably provided by this utilization of glucose. However, the endogenous substrates of the plucked fibers are apparently sufficient to sustain protein synthesis *in vitro* for a limited period. When NaF was present in the medium, the incorporation of ^{14}C-leucine was further diminished, presumably due to the inhibition of both protein synthesis and glycolysis (17).

During incubation the wool root proteins became progressively less soluble in 8M urea and were completely urea-resistant after 24 hours' incubation. This resulted in the presence of an increasing proportion of incorporated ^{14}C in the urea-insoluble residue. During normal keratinization, sulfhydryl groups are oxidized to form disulfide linkages, thus rendering the fiber insoluble in 8M urea (1). The change in solubility of the wool root proteins of incubated fibers was also probably due to the formation of disulfide cross-linkages in both the bulb and precortex region of the fiber. Thus, the addition of cupric ions to the medium increased the rate of conversion of the proteins to a urea-insoluble form and cupric ions are known to catalyze the oxidation of sulfhydryl groups to disulfides (18). This catalysis during incubation was more likely chemical than enzymatic in nature as it occurred more rapidly at higher temperatures and was unaffected by a metabolic inhibitor such as NaF. The presence of a metal-complexing agent such as EDTA presumably lowered the concentration of copper, which is normally present in small amounts within the fiber (19, 20) and thus decreased the rate of formation of urea-insoluble material. Copper deficiency in sheep also retards keratinization *in vivo* by delaying the oxidation of sulfhydryl groups, and this process is likewise

thought to depend on the catalytic action of copper (19, 21, 22, 23).

Studies of the nature of the labeled proteins synthesized *in vitro* were confined to preparations obtained after 1 to 6 hours' incubation, when only a small proportion of the incorporated ^{14}C was present in the 8M urea-insoluble fraction. Electrophoretic separations showed that the ^{14}C-leucine was mainly incorporated into low-sulfur proteins. A large proportion of this ^{14}C was present in the region LS 7–8 (Fig. 9) which corresponded to the two major low-sulfur protein components (24). The small amount of ^{14}C-leucine present in the region of the high-sulfur proteins was in fact comparable to the relatively small proportion (about 10%) of leucine in the high-sulfur fraction from wool (25). The labeled material remaining at the gel origin probably represented low-sulfur proteins rendered insoluble due to freeze-drying (24).

Previous investigations have shown that low-sulfur proteins of wool are mainly synthesized in the bulb region of the follicle (3). Hence, the presence of radioactivity principally over the bulb region of incubated fibers also indicated that most of the ^{14}C-leucine had been incorporated into low-sulfur proteins. Although the *in vitro* incorporation of ^{14}C-leucine may possibly have been affected by the changes in stability of the wool roots, the results are nevertheless comparable to those from *in vivo* labeling experiments. After injection of labeled leucine, de Bersaques (26) found that the radioactivity was confined essentially to the low-sulfur fractions (α- and β-keratose) and was detected in the lower part of the follicle.

From these investigations it can be concluded that despite the changes which occurred during incubation, the plucked wool fibers retained their capacity to synthesize proteins *in vitro*. Furthermore, these proteins resembled those synthesized *in vivo*. By modification of the incubation conditions it may be possible to maintain the "unkeratinized" state of the wool root. The formation of labeled low-sulfur keratin proteins by plucked fibers incubated in the presence of ^{14}C-leucine demonstrates the feasibility of using such a simple *in vitro* system for future studies on the mechanisms of keratin biosynthesis.

The authors wish to thank Mr. C. A. Maxwell for invaluable technical assistance and Dr. R. M. Clarke for many helpful discussions.

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